Role of scaffolds in MAP kinase pathway specificity revealed by custom design of pathway-dedicated signaling proteins

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Background: Signal transduction pathways with shared components must be insulated from each other to avoid the inappropriate activation of multiple pathways by a single stimulus. Scaffold proteins are thought to contribute to this specificity by binding select substrates.

Results: We have studied the ability of scaffold proteins to influence signaling by the yeast kinase Ste11, a MAPKKK molecule that participates in three distinct MAP kinase pathways: mating, filamentation, and HOG. We used protein fusions to force Ste11 to associate preferentially with a subset of its possible binding partners in vivo, including Ste5, Ste7, and Pbs2. Signaling became confined to a particular pathway when Ste11 was covalently attached to these scaffolds or substrates. This pathway bias was conferred upon both stimulus-activated and constitutively active forms of Ste11. We also used membrane-targeted derivatives of the mating pathway scaffold, Ste5, to show that stimulus-independent signaling initiated by this scaffold remained pathway specific. Finally, we demonstrate that loss of pathway insulation has a negative physiological consequence, as nonspecific activation of both the HOG and mating pathways interfered with proper execution of the mating pathway.

Conclusions: The signaling properties of these kinase fusions support a model in which scaffold proteins dictate substrate choice and promote pathway specificity by presenting preferred substrates in high local concentration. Furthermore, insulation is inherent to scaffold-mediated signaling and does not require that signaling be initiated by pathway-specific stimuli or activator proteins. Our results give insight into the mechanisms and physiological importance of pathway insulation and provide a foundation for the design of customized signaling proteins.

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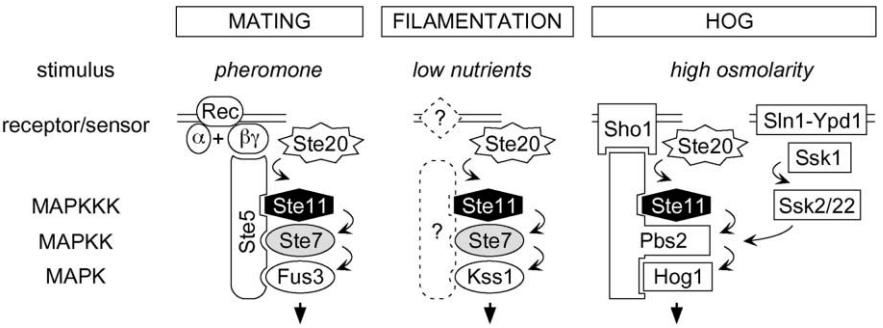
Background

Cellular responses to external cues are often mediated by signal transduction pathways that utilize mitogen-activated protein (MAP) kinase cascades [1], in which signaling proceeds via sequential activation of a MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). In the budding yeast *Saccharomyces cerevisiae*, the Ste11 MAPKKK functions in at least three separate signaling pathways [2]: mating, filamentous growth, and the high-osmolarity glycerol (HOG) response (Figure 1). While these pathways share a common signaling component, each stimulus activates only a single pathway, a phenomenon termed "pathway insulation". Ultimately, each pathway is controlled predominantly by a distinct MAPK: Fus3 for mating, Kss1 for filamentation, and Hog1 for HOG [2, 3]. Therefore, Ste11 must be activated by a mechanism that allows the stimulus to dictate which MAPK becomes activated. The identification of pathway-specific scaffold proteins potentially offers a molecular basis with which to understand this insulation.

A growing number of MAP kinase cascades appear to associate with scaffold proteins [1, 4, 5]. A founding example is the yeast Ste5 protein [6–8], which binds multiple kinases in the mating pathway (Figure 1). In the HOG pathway, the protein Pbs2 is a MAPKK that also functions as a scaffold protein by binding both an upstream MAPKKK, Ste11, and its downstream MAPK, Hog1 [9]. Recently, mammalian proteins that lack sequence similarity to Ste5 but share analogous multikinase binding properties have also been identified [10, 11]. These scaffold proteins appear to perform multiple signaling functions. First, both yeast and mammalian scaffolds increase the efficiency of signal propagation through the kinase cascade [10–13]. Second, they often serve as an adaptor for kinase cascade activation by linking kinases to receptor/

Figure 1



A schematic diagram of pathways involving Ste11. In the HOG pathway, Ste11 functions in one of two redundant branches [9]. In addition to binding kinases, both Ste5 and Pbs2 bind membrane proteins [14, 17]. Membrane recruitment of Ste5 by Gβγ (βγ) is implicated in the activation of the mating pathway [15]; recent evidence suggests that the interaction of Pbs2 with the transmembrane protein Sho1 may play a similar role [16, 40]. Note that Ste20 can function in all three pathways as the activator of Ste11; the suggestion that stimuli bring select substrates to Ste20 [15] may explain how Ste20 avoids cross-talk. Whether a scaffold exists for the filamentation pathway is unknown, but Ste5 is not thought to fulfill this function, as it is not required for filamentation [2, 3]. This figure may oversimplify the distinction between Fus3 and Kss1, as recent evidence suggests that, while Fus3 may dominate the mating response, Kss1 may still participate even in the wild-type cells [22, 41, 42], and not only in cells lacking Fus3, as was previously suggested [21]. Rec, pheromone receptor.

sensor molecules (Figure 1); for example, Ste5 links activation of Ste11 by Gβγ [12, 14, 15], whereas Pbs2 links Ste11 activation to Sho1 [16, 17]. Relatedly, the mammalian scaffold JIP-2 can bind a neuronal transmembrane receptor, ApoER2 [18]. It is expected, though not proven [5], that these interactions between sensors and scaffolds determine which kinase cascade becomes activated by a given stimulus.

Third, because of their ability to link multiple components of a specific pathway, scaffolds are expected to play a key role in insulating the pathway's signaling activity, by assembling unique signaling complexes [5, 19]. While logical, there has been little direct evidence for this third role [3–5, 20]. Here, we substantiate the proposed pathway insulation role with evidence that scaffold-associated signaling in vivo is biased toward activation of downstream molecules that bind to the same scaffold. The results provide mechanistic insight into how scaffolds ensure signaling specificity and also demonstrate that this effect is physiologically important. In addition, we have developed a potentially generalizable experimental approach that allows the conversion of a multifunctional signaling protein into a pathway-specific form.

Results

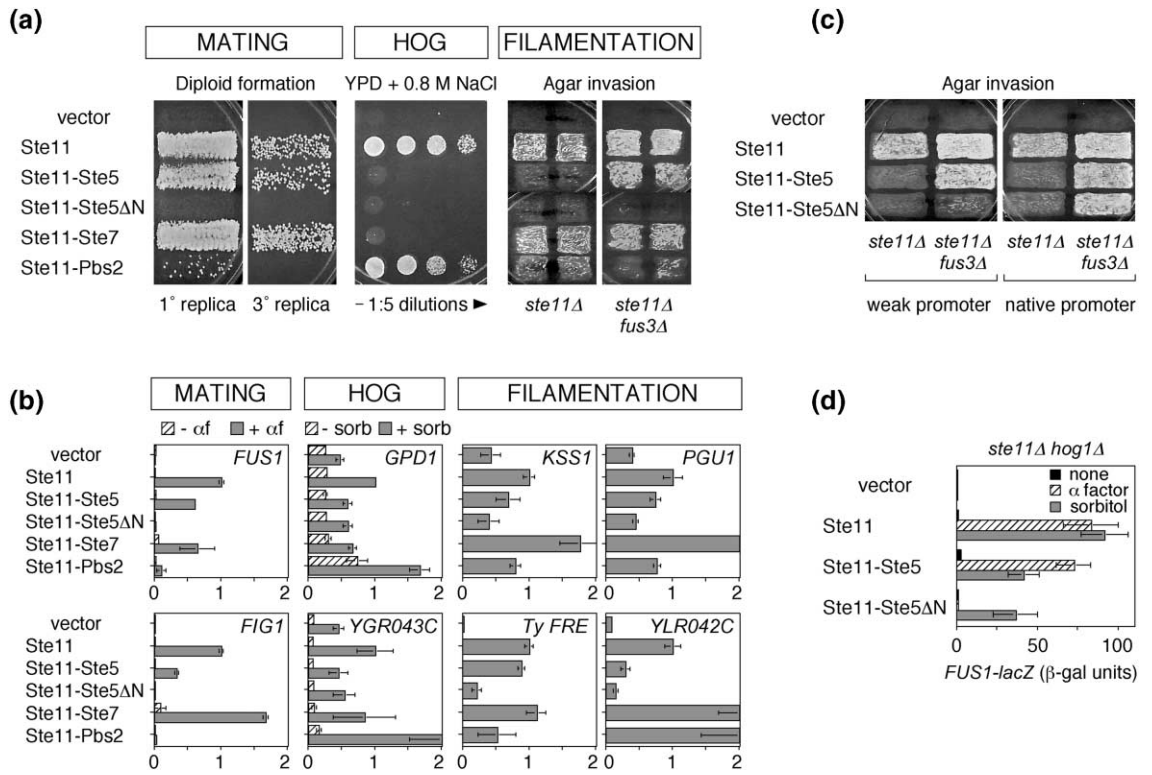
A common kinase becomes pathway dedicated when linked to binding partners

To address whether scaffolds normally partition Ste11 into discrete, pathway-dedicated signaling complexes, we used protein fusions to test if covalent attachment to a single scaffold, or to a substrate normally presented by that scaffold, could create a pathway-specific form of the kinase. In essence, this strategy attempts to illuminate the properties of a kinase when it is associated with a unique signaling complex, simply by forcing the complex to remain associated. Constructs were designed (see the Materials and methods) to encode fusions of three different proteins to the C terminus of Ste11: Ste5, the mating-specific scaffold protein; Pbs2, the MAPKK and scaffold protein in the HOG pathway; and Ste7, the MAPKK substrate of Ste11 shared by the mating and filamentation pathways. We also constructed a fusion between Ste11 and Ste5ΔN [15], which includes the kinase binding domains of Ste5 but lacks an N-terminal domain that mediates kinase activation in response to Gβγ. These derivatives were expressed in *ste11*Δ mutant cells and were analyzed for function in the mating, HOG, and filamentation pathways. Because some of the fusions were mildly toxic (see the Materials and methods), most results shown here analyze the fusions when expressed from a weak promoter (the glucose-repressed *GAL1* promoter), though results were similar when the native *STE11* promoter was used and are provided as Supplementary material available with this article online (Figure S1).

The ability of Ste11 to participate in these three different pathways was indeed affected by the fusions (Figure 2a). For the mating pathway, the fusions of Ste11 to Ste5 and Ste7 retained the ability to complement the sterility of *ste11*Δ cells, whereas the fusion to Pbs2 functioned poorly. Conversely, for the HOG pathway, the fusion to Pbs2 allowed growth on high-osmolarity medium, while the fusions to Ste5 and Ste7 functioned poorly. Finally, for the filamentation pathway, which requires Ste7, but neither Ste5 nor Pbs2, the fusion to Ste7 promoted filamentous agar invasion, whereas fusions to Ste5 and Pbs2 exhibited reduced function (Figure 2a, *ste11*Δ panel). Thus, each fusion converted Ste11 from a common kinase into one that acts preferentially in the pathway of the fusion partner.

Although both the filamentation MAPK Kss1 and the mating MAPK Fus3 are capable of binding the mating scaffold, Ste5, it is thought that pheromone signaling via Ste5 favors the mating pathway because Fus3 outcompetes [21] or antagonizes [22] Kss1. Thus, to address why

Figure 2

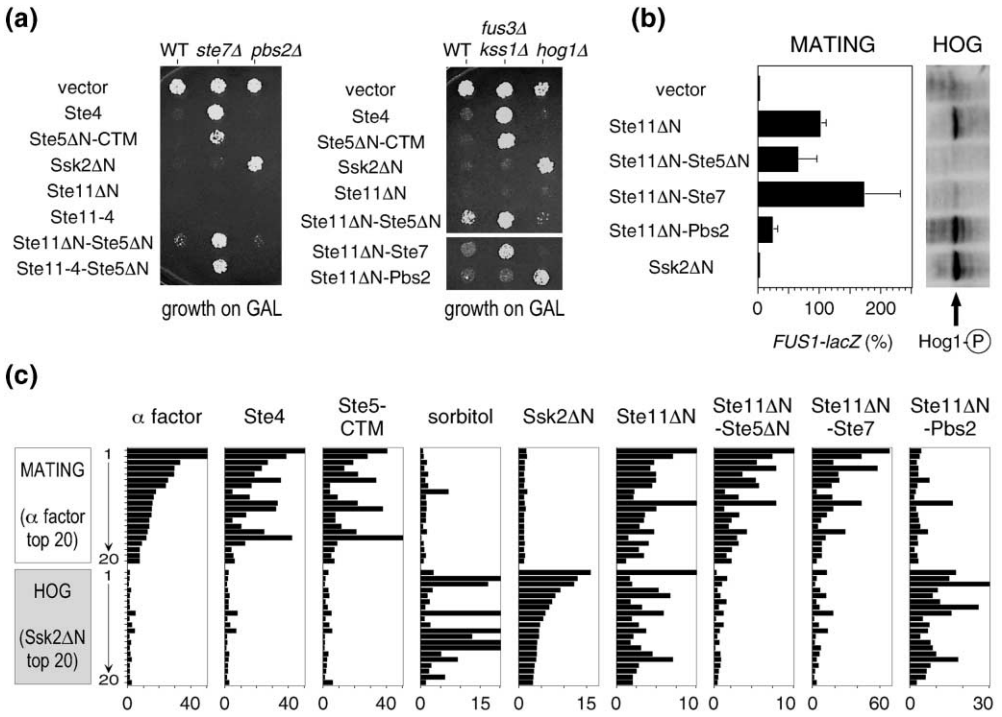


Pathway-biasing effects of fusions to Ste11. **(a)** Comparison of the Ste11 fusion derivatives for mating ability, agar invasion, and growth on high-osmolarity medium (see Materials and methods). Strains: PPY890 (*ste11*Δ; left), FP75 (*ste11*Δ *ssk2*Δ *ssk22*Δ; middle), PPY1057 (Σ1278b *ste11*Δ; right), and PPY1157 (Σ1278b *ste11*Δ *fus3*Δ, far right). Plasmids expressed the indicated Ste11 derivatives from a weak promoter (the glucose-repressed *GAL1* promoter; see Materials and methods); results were similar when expressed from the *STE11* promoter (see Figure S1a). Mating results were confirmed by quantitative assays: for vector, Ste11, Ste11-Ste5, Ste11-Ste5ΔN, Ste11-Ste7, and Ste11-Pbs2, mating efficiencies were 0.00001%, 26%, 31%, 0.00001%, 8.0%, and 0.016%, respectively, when expressed from the glucose-repressed *GAL1* promoter, and were 0.00001%, 90%, 46%, 0.00003%, 139%, and 1.9%, respectively, when expressed from the *STE11* promoter. **(b)** Transcriptional reporter data. Promoters of the indicated genes controlled *lacZ*; β-galactosidase activity was measured in the absence of any stimulus for filamentation reporters or in the absence (−) and presence (+) of 10 μM α factor (αf) or 1 M sorbitol (sorb) for 2 hr. Results (mean ± SD of 4–6 measurements) are shown relative to unfused Ste11 (=1) for each reporter. Note that HOG reporters yield significant sorbitol induction independent of Ste11. The *FUS1* graph exemplifies results using *STE11* promoter-driven constructs; all others use the same weak promoter constructs assayed in (a). Values of off-scale bars: 2.2 (*YGR043C*), 5.6 (*PGU1*), 2.5 (*YLR042C*, Ste11-Ste7), and 2.5 (*YLR042C*, Ste11-Pbs2). The basal signal for mating reporters was elevated for the Ste11-Ste7 fusion by 46-fold (*FUS1*) and 21-fold (*FIG1*). Strains were as in (a) (except for the *FIG1* graph, which used PPY1097), carrying appropriate reporter plasmids (see Materials and methods). **(c)** Comparison of agar-invasion ability of Ste11 fusions to Ste5 and Ste5ΔN when expressed from the glucose-repressed *GAL1* (weak) promoter or the *STE11* (native) promoter. Strains were as in (a). **(d)** The Ste11-Ste5ΔN fusion can respond to sorbitol-induced cross-talk, but not to α factor in a *ste11*Δ *hog1*Δ strain. Strain PPY1083 (*ste11*Δ *hog1*Δ *FUS1-lacZ*) contained plasmids expressing the indicated Ste11 fusions from the *STE11* promoter and was treated with no stimulus (none), 5 μM α factor, or 1 M sorbitol for 4 hr. Bars indicate the mean ± SD of four measurements. Results were similar in a *ste11*Δ *pbs2*Δ strain, but no cross-talk occurred for any construct in strains with an intact HOG pathway (data not shown).

Ste11-Ste5 is reduced for filamentation function, we tested whether this function could be restored by the elimination of Fus3. Indeed, agar invasion was restored specifically to the Ste11-Ste5 fusion and not to Ste11-Pbs2 (Figure 2a, far right panel). Therefore, while Ste5 is clearly dispensable for filamentation [3, 23], these results show that association with Ste5 causes Ste11 to favor the mating pathway over the filamentation pathway, at least in part because it leads to preferential activation of Fus3-dependent signaling.

To quantify pathway participation by the Ste11 derivatives, we measured expression of pathway-specific transcriptional reporters (Figure 2b). Induction of mating reporters was best retained by Ste11-Ste5 and Ste11-Ste7 and was severely reduced for Ste11-Pbs2. Conversely, induction of HOG reporters was best retained (and actually was made hyperactive) by Ste11-Pbs2 and was reduced by the fusions to Ste5 or Ste7. The behavior of Kss1-regulated filamentation reporters [24] was more complex but showed some general trends, with activation

Figure 3



Fusions route pathway flux by constitutively active Ste11. **(a)** Growth arrest by galactose-induced expression of the indicated proteins in wild-type (WT) or mutant strains. Transformants (500 cells) were spotted onto selective medium containing raffinose and galactose (GAL) and were incubated for 3 days at 30°C; fusions to Ste7 and Pbs2 were rescued incompletely by the gene deletions and thus are shown less diluted (12,500 cells/spot) after 5 days. Strains, from left: PPY1114, PPY1098, PPY1131, PPY398, PPY1139, and PPY1087. **(b)** *FUS1-lacZ* induction and Hog1 phosphorylation in strain FP75 (*ssk2*Δ *ssk22*Δ *ste11*Δ), following galactose-induced expression of the indicated proteins. *FUS1-lacZ* assays used the reporter p3058-T, assayed after 3 hr in 2% galactose; bars indicate the mean + SD (n = 8), relative to the Ste11ΔN signal (=100%). Similar results were seen in other strains (e.g., see Figure 4b legend). Hog1 tyrosine phosphorylation was monitored 1 hr after galactose induction by immunoblotting with a phospho-specific p38 antibody (New England Biolabs); the top of the gel was oriented toward the left. The results are representative of four experiments. **(c)** Transcriptional induction of representative gene sets monitored using DNA microarrays. The mating and HOG gene sets were the 20 genes most strongly induced by α factor and Ssk2ΔN, respectively (see Materials and methods). The ratio of expression (induced/uninduced) is shown for the genes in each set. Cells were treated with 50 nM α factor for 30 min or 1 M sorbitol for 15 min, or with 2% galactose for 3 hr to induce synthesis of the indicated protein. See Figure S2 for additional analysis.

by Ste11-Ste5 being reduced to varying extents and Ste11-Ste7 being hyperactive for most reporters. Ste11-Pbs2 was marginally affected for two filamentation reporters (*KSS1* and *PGU1*) and was more reduced for another (Ty FRE); yet, it was hyperactive for a fourth (*YLR042C*), which was subsequently found to also be induced by osmotic stimuli (see Figure 3c). While the filamentation reporters showed complex and intermediate sensitivity to the fusions (see the Supplementary material), the overall results with all the reporters lend quantitative support to the notions gained from the plate assays: that association of Ste11 with a pathway-specific protein causes it to function best in that pathway and to become less available for other pathways. In addition, they uncover hyperactivity (which, notably, was pathway specific) as a common result of expressing both MAPKKK and MAPKK as a single polypeptide; separate polypeptides may be better suited to dampen signaling noise from chance fluctuations in kinase activity [25].

The Ste11-Ste5ΔN fusion was defective in all three pathways (Figure 2a,b). Because Ste5ΔN lacks Gβγ binding ability, this fusion may lock Ste11 into a form committed to the mating pathway but unactivatable by pheromone. Its behavior demonstrates that sequestration of Ste11 from nonmating pathways is independent of successful mating signaling. Two special situations revealed that Ste11-Ste5ΔN retains the capacity to signal. First, filamentation activity of Ste11-Ste5ΔN could be restored by *FUS3* deletion, though it remained weaker than Ste11-Ste5, as revealed by expression from weak versus native promoters (Figure 2a,c), suggesting that the filamentation induced by Ste11-Ste5 in *fus3*Δ cells may be enhanced by basal signaling from Gβγ [21]. Second, Ste11-Ste5ΔN was com-

petent to perform scaffold-independent signaling. In *hog1*Δ mutants, the absence of a negative-feedback loop allows osmotic stimuli to induce cross-talk into the mating pathway [26]; here, by unknown means, Ste11 activation and signal transmission require neither Pbs2 nor Ste5, somewhat analogous to mutationally activated Ste11 forms [9, 27–29]. In this setting, Ste11-Ste5ΔN could activate the mating reporter *FUS1-lacZ*, but only in response to sorbitol and still not in response to pheromone (Figure 2d), whereas Ste11 and Ste11-Ste5 responded to both stimuli. Thus, contrary to the normal osmotic response (see above), in which Ste11 must bind Pbs2, fusion to Ste5 or Ste5ΔN does not block Ste11 from mediating the osmotic cross-talk response, consistent with its scaffold independence. Furthermore, these data show that Ste11-Ste5ΔN is a functional kinase, but one whose properties have been altered to allow mating pathway activation only in response to an artificial cross-talk stimulus.

Pathway preferences of constitutively active signaling proteins

In theory, the pathway-biasing effects of the fusion partners could affect which stimulus can activate Ste11, which substrates Ste11 phosphorylates, or both. To test directly whether substrate choice can be specified, we bypassed the stimulus requirement by making fusions to constitutively active versions of Ste11 (expressed from a galactose-inducible promoter): Ste11ΔN, which lacks an N-terminal inhibitory domain [28], and Ste11-4, which harbors a kinase domain mutation rendering it insensitive to the inhibitory domain [27, 29]. For fusions involving the mating scaffold, Ste5, we used the Ste5ΔN derivative [15] in order to exclude any contribution from the Gβγ binding and dimerization abilities of Ste5 to the signaling properties of the resulting fusion. In addition, these activated Ste11 derivatives were compared with membrane-targeted Ste5 forms, Ste5-CTM and Ste5ΔN-CTM [15], which also activate Ste11 constitutively but appear to do so in a pathway-specific manner, as shown below.

One test of pathway specificity (Figure 3a) measured whether growth arrest activated by the galactose-induced product could be relieved by the inactivation of individual pathways [9]. For example, overproduction of Ste4 (Gβ) activates only the mating pathway and not the HOG pathway, as evidenced by growth arrest that was relieved by inactivation of the mating pathway (*ste7*Δ or *fus3*Δ *kss1*Δ), but not the HOG pathway (*pbs2*Δ or *hog1*Δ). Importantly, this pattern was mimicked by a membrane-targeted Ste5 derivative (Ste5ΔN-CTM), which signals independent of pheromone and Gβγ [15], indicating that scaffold-mediated activation can maintain pathway specificity. A reciprocal pattern was observed with Ssk2ΔN, an activated form of the HOG pathway kinase Ssk2 [9]. Unlike these pathway-specific activators, Ste11ΔN and Ste11-4 cause growth arrest that is not relieved by single pathway lesions [9]; but, when attached to Ste5ΔN, they became diverted away from the HOG pathway, since growth arrest is now relieved by inactivation of the mating pathway alone (Figure 3a). Similar tests indicated that attachment to Ste7 or Pbs2 caused Ste11ΔN to become biased toward the mating or HOG pathway, respectively (Figure 3a).

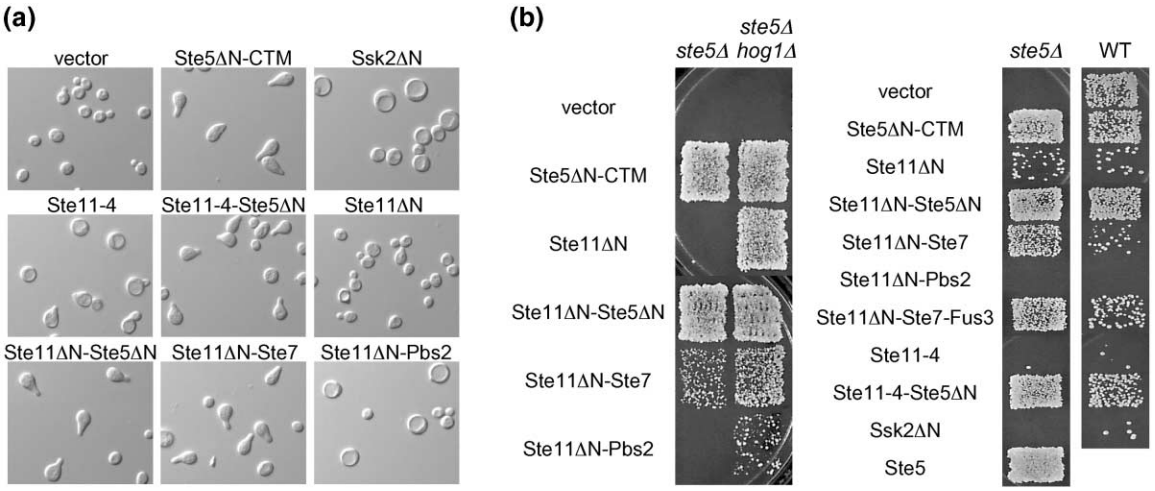
To directly measure pathway activation, we assayed transcriptional induction of *FUS1-lacZ* and tyrosine phosphorylation of the Hog1 MAPK (Figure 3b). When attached to Ste5ΔN or Ste7, Ste11ΔN retained the ability to induce *FUS1-lacZ* but was severely reduced for Hog1 phosphorylation. Conversely, when attached to Pbs2, Ste11ΔN displayed reduced (though not eliminated) *FUS1-lacZ* transcription but remained competent to induce Hog1 phosphorylation. These results are in agreement with the growth-arrest assays. Together, they demonstrate that the fusion partners can route the flow of signal from a preactivated kinase to favor either the mating or HOG pathway.

We also examined transcriptional profiles using DNA microarrays in order to compare relative levels of activity through different pathways using a single assay. These profiles were determined for a variety of experimental treatments, and detailed analysis is provided as Supplementary material (Figure S2). For simplicity, we focus here on a subset of experiments and on the relative induction of 2 representative gene sets (Figure 3c), each containing 20 genes induced by either the mating or HOG pathways (see the Materials and methods). The mating gene set was activated preferentially when cells were stimulated by α factor, overproduction of Ste4, or expression of membrane-targeted Ste5, whereas the HOG gene set was activated preferentially by stimulation with sorbitol or expression of Ssk2ΔN. In contrast to these pathway-specific profiles, Ste11ΔN did not show preference for either pathway and instead induced both sets of genes. When fused to either Ste5ΔN or Ste7, however, Ste11ΔN became biased for the mating gene set, whereas fusion to Pbs2 caused bias for the HOG gene set. These results clearly indicate that the fusion partners impose pathway preference upon the active kinase.

Loss of insulation interferes with execution of the mating pathway

The physiological importance of pathway insulation was revealed by examining two aspects of whole-cell physiology: morphology and mating ability. Cells exposed to mating pheromone develop a pear-shaped morphology called a "shmoo" [2]. This morphology was well mimicked by activation of the mating pathway using Ste5ΔN-CTM, whereas persistent activation of the HOG pathway by Ssk2ΔN produced a population of cells that were enlarged and round, with prominent vacuoles (Figure 4a). Ste11ΔN and Ste11-4 were less effective than Ste5ΔN-CTM at generating a uniform shmoo morphology (Figure 4a) and

Figure 4



Physiological effects of pathway insulation. **(a)** Morphology. Representative fields of cells are shown 4 hr after galactose (2%) induction of the indicated products in a wild-type strain (PPY640); mating and *FUS1-lacZ* results for these cells are reported in (b). Note that a small fraction of cells will be unaffected, due to plasmid loss. **(b)** Mating ability. Congenic *ste5*Δ and *ste5*Δ *hog1*Δ strains (PPY655 and PPY1085) or congenic *ste5*Δ and wild-type (WT) strains (PPY858 and PPY640) harboring galactose-inducible constructs were mated overnight on SC/raffinose/galactose plates. Observing inhibition of WT mating necessitated selecting for the *URA3* plasmid in the diploid progeny, by using the *ura3* partner strain PPY181; otherwise, the effect was obscured by uninhibited mating of haploids that lost the plasmid (data not shown). All other matings used partner PT2α. Poor mating by HOG-activating constructs is distinguishable from cell lethality, as it was observed when monitoring either diploid or zygote formation during brief quantitative mating assays in which the unmated haploid cells remained viable upon return to glucose medium (P.M.P., R.L., and K.H., unpublished data). Some strain background differences were noted: rescue of *ste5*Δ by Ste11ΔN was worse in the 381G background (left) than in the W303 background (right), and rescue by *hog1*Δ was more evident in 381G (left) than in W303 (data not shown). *FUS1-lacZ* induction, measured after 3 hr of galactose induction and normalized to Ste11ΔN (=100), was as follows (from top to bottom): in PPY655 (*ste5*Δ, far left): 0, 216, 100, 184, 189, 7; in PPY858 (*ste5*Δ, right): 0, 86, 100, 43, 147, 12, 156, 102, 99, 0, 1; in PPY640 (WT, far right): 0, 118, 100, 44, 205, 13, 173, 104, 108, 0 (averaged from three or more measurements).

instead gave a heterogenous population, likely due to dual activation of mating and HOG pathways. Consistent with this view, attachment of Ste5ΔN to either activated kinase allowed for more-uniform shmoo formation, as did attachment of Ste7 to Ste11ΔN. In contrast, attachment of Pbs2 to Ste11ΔN did not favor shmoo formation, but rather favored the enlarged, round cell morphology. Thus, the unrestricted kinases induce a heterogeneous morphogenetic response, but association with pathway-specific scaffolds and kinases tips the signaling balance such that cell shape changes become determined by a specific pathway.

Efficient completion of the mating process also required pathway insulation. We compared the constitutive signaling constructs for their ability to promote efficient mating by testing how well they rescued the sterility of *ste5*Δ mutants. Ste11ΔN was worse at stimulating mating in *ste5*Δ cells than Ste5ΔN-CTM (Figure 4b), despite inducing comparable levels of *FUS1-lacZ* (see Figure 4b legend). This poor mating was dramatically improved when Ste11ΔN was steered away from the HOG pathway by attachment to Ste5ΔN or Ste7; in contrast, attachment to Pbs2 had the opposite effect. Eliminating HOG pathway signaling by deletion of the *HOG1* gene also improved mating by Ste11ΔN (Figure 4b, left panel). These observations suggest that the poor mating of activated Ste11 proteins reflects their promiscuous activity, rather than a functional deficiency. In support of this interpretation, activated Ste11 proteins inhibited mating of wild-type cells, and this phenotype was suppressed by fusions that disfavor HOG pathway activation (Figure 4b, far right panel). In either assay, fusion to Ste7 was slightly less effective than fusion to Ste5ΔN at improving mating, suggesting incomplete restriction to the mating pathway; this was improved somewhat by adding Fus3 to the fusion, creating Ste11ΔN-Ste7-Fus3 (Figure 4b, right panels). Overall, these mating and morphological results underscore the physiological significance of pathway insulation. While Ste11ΔN activates mating signaling, its ability to simultaneously activate HOG signaling interferes with the proper execution of the mating response. In contrast, Ste5-mediated signaling (either by Ste5ΔN-CTM or by wild-type Ste5 in response to pheromone) ensures pathway insulation and thereby allows cells to assume the appropriate morphology and conjugate efficiently.

Discussion

Scaffolds direct the flow of intracellular signaling

Prior studies on MAP kinase scaffolds have emphasized their ability to bind select sets of kinases and to enhance

signaling efficiency, occasionally with preferential effect on specific kinases [10, 11, 13]. Here, we have used a novel protein-fusion approach to test the ability of scaffolds to selectively route signaling traffic, and we present several new observations.

The ability to confine a common kinase to a particular pathway by covalent attachment to a pathway-specific scaffold (Figure 2) implies that scaffolds organize signal transduction proteins into distinct, pathway-dedicated signaling complexes. That signaling by a constitutively active kinase can be similarly routed (Figure 3) indicates that scaffolds can actively channel signaling by discriminating among downstream substrates. Interestingly, fusion of a kinase to a downstream substrate can, in some respects, mimic fusion to its scaffold (Figures 2 and 3), suggesting that scaffolds promote specificity in part by presenting a preferred substrate in high local concentration. This ability of a scaffold to bind both kinase and substrate may cooperate with separate kinase-substrate interactions to ensure optimal signal transmission [30]. While scaffolds can enhance signaling, our results suggest that scaffolds can also sequester common molecules into complexes that exclude them from other pathways. The signaling properties of our kinase fusions are also relevant to the "switch-like" behavior of some kinase cascades [25], which may be abrogated by scaffolds [4, 31] or by single polypeptides containing two or more kinases. Finally, we created an altered form of a common kinase (Ste11-Ste5ΔN) that is dedicated to the mating pathway but can only activate this pathway in response to an artificial cross-talk stimulus (Figure 2d), providing an example of using scaffolds for signal transduction pathway rewiring and attesting to the potential to do so for desirable or therapeutic outcomes.

Signaling initiated by a scaffold protein maintains pathway specificity

Our membrane-targeted Ste5 derivatives (Ste5ΔN-CTM and Ste5-CTM; [15]) activate the mating pathway independently of the normal stimulus (pheromone) and activator protein (Gβγ). Here, we show that they maintain specificity for the mating pathway, using four criteria encompassing both specific signaling events and overall physiological response: growth arrest behavior, transcriptional profile, polarized cell morphology, and conjugation efficiency (Figures 3 and 4). In total, the results demonstrate that pathway insulation can be inherent to scaffold-mediated signaling and does not require that signaling be initiated by pathway-specific activators upstream. Ste5-CTM and Ste5ΔN-CTM are especially well suited for studying this intrinsic fidelity, because of their extreme insensitivity to input from pheromone and Gβγ [15], with Ste5ΔN-CTM being particularly insensitive because it lacks the N-terminal Gβγ binding domain. Importantly, the remaining "upstream" component required for activation by membrane-targeted Ste5 is the PAK-family kinase Ste20 [15], which functions in all pathways under consideration here (see Figure 1). Therefore, Ste5 itself can specify that a common activating kinase, Ste20, will stimulate a unique pathway. It is expected that coupling the appropriate set of kinases to a specific stimulus occurs via the adaptor properties of scaffolds. Consistent with this expectation, the mating pathway becomes responsive to osmotic stimuli when the Sho1 binding domain from Pbs2 is attached to Ste5 (R.L. and P.M.P., unpublished data).

Physiological importance of pathway insulation

Our data demonstrate that loss of insulation can negatively impact the outcome of the mating MAP kinase pathway (Figure 4), including appropriate cell polarization. The importance of ensuring insulation between mating and HOG pathways may arise in part because of their different temporal behaviors. The conjugation process requires that the mating pathway be activated persistently, and cells can stay arrested with high gene-induction levels for many hours. In contrast, activation of the HOG pathway is ordinarily transient, with the Hog1 kinase becoming inactivated within 20–30 min [17, 32], even in the continued presence of high osmolarity (presumably reflecting osmotic equalization). Thus, cross-talk from uninsulated mating pathway signaling into the HOG pathway would not only be inappropriate, but the persistence of the signaling would be counter to normal HOG pathway behavior. Indeed, the enlarged round cell morphology (Figure 4a) is not a normal high-osmolarity response but appears to result from persistent HOG pathway activation (likely bypassing a negative-feedback loop [26]). The ability of the fusion partners to dampen inappropriate signaling and thus restore more-physiological mating behavior to the activated Ste11 derivatives makes these fusion constructs useful tools for further study of cell signaling and morphogenesis.

Relevance of fusions to normal signaling properties

To address how the signaling properties of a common kinase (Ste11) are changed by association with a pathway-specific scaffold protein, we examined the most extreme situation: namely, when all Ste11 molecules in the cell were permanently associated with a single scaffold. The results support the notions that scaffolds can contribute to signaling fidelity by preventing their associated kinases from becoming activated by inappropriate upstream stimuli and by preventing them from phosphorylating inappropriate downstream targets, at least while they remain associated. Normally, however, the kinase would have the opportunity to dissociate from the scaffold at some rate. Therefore, we expect that the properties imposed by the permanent association of the fusion partners reflect those that would ordinarily be imposed transiently.

This draws attention to the temporal relationship between kinase activation/inactivation and complex assembly/disassembly. Cross-talk could still occur if a kinase activated

in association with one scaffold could dissociate and then associate with another scaffold while still in active form. Thus, to maintain pathway insulation, kinase inactivation might be either coupled to dissociation or rapid in comparison to dissociation from the scaffold. While a short-lived kinase activity might otherwise lead to inefficient signaling, scaffolds can increase efficiency [10–13, 30] by providing immediate access to a select substrate. In this view, the insulating and "catalytic" [4] functions of scaffolds are interrelated, and the efficacy of insulation becomes dependent on a mechanism for signal termination. Indeed, inactivation of a negative-feedback loop in the HOG pathway allows a persistent signaling state to develop, eliminating both signaling fidelity and scaffold dependence [26].

Experimental conversion of multifunctional proteins into pathway-specific forms

Protein fusion is a straightforward and simple method of localizing two proteins into a functional complex with the potential for broad application. We used this approach to compare the properties of signaling complexes that normally coexist in the cell. Such a comparison would ordinarily require cell lysis, fractionation, and assay in vitro. Our method provides an alternative, in which cell lines that contain only one of multiple original signaling complexes can be generated, thereby allowing for "fractionation" studies at an in vivo, whole-cell level. Other cases in which this approach could be instructive include deciphering the functions of individual cyclin/Cdk complexes [33] or complexes of Rho GTPases and their effectors [34]. A related method was recently used to generate constitutively active MAPKs [35, 36]. While our studies derivatize an individual signaling molecule, a broader application of the protein fusion strategy, or a more general strategy linking proteins through a protein-protein interaction domain, should allow one to focus the activity of many proteins to specific pathways, potentially on a genomic scale. Conceivably, this approach could also provide the basis for a screening method to identify molecules that impart specific roles to multifunctional proteins.

Conclusions

Because fusion of Ste11 to a substrate (Ste7) mimics fusion to a scaffold (Ste5), and fusion to a scaffold predisposes Ste11 to the use of a particular set of downstream substrates, we conclude that scaffold proteins dictate substrate use and promote pathway insulation by presenting a preferred substrate in high local concentration and by excluding other substrates. Moreover, because membrane-targeted Ste5 derivatives maintain pathway specificity, insulation does not require signaling to be initiated by a stimulus (e.g., pheromone) or pathway-specific activators (e.g., Gβγ) but is inherent to scaffold-mediated activation. Ordinarily, adaptor properties of scaffolds are expected to dictate which set of kinases is activated by a specific stimulus. Maintenance of pathway insulation is important for the efficient execution of at least one MAPK pathway. Finally, the fusion approach used here, or alternative protein linkage approaches, may be generally applicable to other multifunctional proteins as a way of restricting them to only a subset of their functions.

Materials and methods

Yeast strains

W303 background strains (*ade2 his3 leu2 trp1 ura3 can1*): PPY398 (**a** wild-type), PPY640 (**a** *FUS1::FUS1-lacZ::LEU2*), PPY858 (**a** *ste5::ADE2 FUS1::FUS1-lacZ::LEU2*), PPY890 (**a** *ste11::ADE2 FUS1::FUS1-lacZ::LEU2*), PPY1083 (**a** *ste11::ADE2 hog1::hisG FUS1::FUS1-lacZ::LEU2*), PPY1087 (**a** *hog1::hisG*), PPY1097 (**a** *ste11::ADE2*), PPY1098 (**a** *ste7::ADE2*), PPY1114 (**a** *ADE2*), PPY1131 (**a** *ADE2 pbs2::LEU2*), PPY1139 (**a** *fus3::LEU2 kss1::ura3^{FOA}*), and SO622 (**a** *ssk1::HIS3$^{C.g.}$ ste11::LEU2$^{C.g.}$*). 381G background strains (*cry1 ade2 ade3 his4 leu2 lys2 trp1 ura3 SUP4-3 FUS1::FUS1-lacZ::LYS2*): PPY655 (**a** *ste5::LYS2*) and PPY1085 (**a** *ste5::LYS2 hog1::hisG*). Σ1278b background strains (*his3 leu2 trp1 ura3*): PPY1057 (**a** *ste11::hisG*) and PPY1157 (**a** *ste11::hisG fus3::LEU2*). Other strains used were FP75 (α *his3 leu2 trp1 ura3 ssk2::LEU2 ssk22::LEU2 ste11::HIS3*), PPY181 (α *his7 lys9 ura3 can1 cyh2*), and PT2α (α *hom3 ilv1 can1*).

Plasmid use and general descriptions

Fusions to wild-type, full-length *STE11* were placed under the control of both the native *STE11* promoter and the *GAL1* promoter. Although similar when using the *STE11* promoter, most results presented here used reduced expression conditions in which *GAL1* promoter-controlled constructs were assayed in repressive glucose media, because the fusions to Ste7 and Pbs2 mildly slowed growth when expressed from the *STE11* promoter (which was alleviated by deletion of *FUS3* and *HOG1*, respectively [data not shown], indicating hyperactivity in the pathway of the fusion partner; see reporter data in Figure 2c). In the Σ1278b background used for filamentation assays, this effect was so severe that the Ste7 and Pbs2 fusions could not be tested for filamentation function when expressed from the *STE11* promoter. Therefore, to allow comprehensive analysis and to ensure that the results were not affected by growth rate, we tested all fusions as expressed from a weak promoter, the glucose-repressed *GAL1* promoter, which eliminated the toxic effect. Confirmation of the results using *STE11* promoter-controlled fusions is shown in Figure S1. Also, the *FUS1* panel of Figure 2b shows an example of transcription results with *STE11* promoter-driven fusions, and, in Figure 2c, both expression methods are used to compare Ste11-Ste5 and Ste11-Ste5ΔN for agar invasion in *ste11Δ fus3Δ* cells. In general, residual function in disfavored pathways was greater when expressed from the *STE11* promoter than from the glucose-repressed *GAL1* promoter.

Fusions to *STE11-4* and *STE11ΔN* were controlled by the *GAL1* promoter and were studied only upon galactose induction. Immunoblotting with anti-Ste11 serum [28] confirmed that expression levels were roughly comparable for the various Ste11ΔN fusions after galactose induction (data not shown), though we were unable to detect Ste11 fusions that were not overexpressed.

All Ste11 fusion and pathway-inducing plasmids were CEN *URA3*. Plasmids pGAL-SSK2ΔN [17], pL19 [37], and pRS316 [38] were previously described; others were constructed as described in the Supplementary material. Plasmid names are as follows, with their encoded products in parentheses. For experiments involving fusions to wild-type Ste11 expressed from the *STE11* promoter (Figures 2b, *FUS1*; 2c,d; and S1a), plasmids were: pRS316 (vector), pS11 (Ste11), pS11.S5 (Ste11-Ste5), pS11.S5ΔN (Ste11-Ste5ΔN), pS11.S7 (Ste11-Ste7), and pS11.PB (Ste11-Pbs2). For experiments involving fusions to wild-type Ste11 expressed from the glucose-repressed *GAL1* promoter (Figures 2a–c and S1b), plasmids were: pRD53* (vector), pG11 (Ste11), pG11.S5 (Ste11-

Ste5), pG11.S5ΔN (Ste11-Ste5ΔN), pG11.S7 (Ste11-Ste7), pG11.PB (Ste11-Pbs2), pU-GS5ΔN.S11 (Ste5ΔN-Ste11), and pU-GS5.S11 (Ste5-Ste11). For galactose-induced experiments (Figures 3 and 4), plasmids were: pRD53* (vector), pL19 (Ste4), pU-GS5ΔN-CTM (Ste5ΔN-CTM), pGAL-SSK2ΔN (Ssk2ΔN), pG11ΔN (Ste11ΔN), pG11-4 (Ste11-4), pG11ΔN.S5ΔN (Ste11ΔN-Ste5ΔN), pG11-4.S5ΔN (Ste11-4-Ste5ΔN), pG11ΔN.S7 (Ste11ΔN-Ste7), pG11ΔN.PB (Ste11ΔN-Pbs2), pG11ΔN.S7.F3 (Ste11ΔN-Ste7-Fus3), and pU-GS5 (Ste5).

Mating, high-osmolarity growth, and agar-invasion assays

For patch mating assays, transformants were patched onto a lawn of mating partner strain PT2α or PPY181, on SC/glucose or SC/raffinose/galactose medium as indicated, and incubated overnight at 30°C, then diploids were selected by replication to minimal medium. After an immediate (1°) replica of the mating plate was made, more-diluted replicas were generated by repeating the replication of the master mating plate twice more, using a fresh velvet each time, to generate 2° and 3° replicas; the 1° and 3° replicas are shown.

Quantitative mating assays were conducted in strain PPY890 (**a** *ste11*Δ) by mixing 5×10^6 transformant cells with 1×10^7 partner cells (PT2α), collecting onto filters, mating on SC/glucose plates at 30°C for 4 hr, then harvesting and plating serial dilutions onto minimal medium to select for diploids. Mating efficiency is defined as the number of diploids expressed as a percentage of input plasmid-containing haploid **a** cells. The results reported (Figure 2 legend) represent averages of duplicate measurements, with all constructs assayed in parallel.

For testing HOG function, transformants were suspended and were serially diluted 5-fold. Aliquots (5 μl; $\sim 3 \times 10^4$ cells in the first spot) were applied onto YPD plates containing 0.8–1.2 M NaCl or 1 M sorbitol. Plates were photographed after incubation at 30°C for 3 days (0.8 M NaCl, 1 M NaCl, or 1 M sorbitol) or 5 days (1.2 M NaCl).

Agar-invasion assays were conducted by patching cells onto YPD plates, using transformants grown on −Ura plates for 3–5 days; the plates were incubated for 30 hr at 30°C, and then nonadherent cells were rinsed off with a gentle stream of distilled water.

Transcriptional reporter assays

β-galactosidase assays were performed as described [15] on exponentially growing cultures, with or without galactose induction or stimulus addition. *FUS1-lacZ* experiments used strains with integrated reporters, except for those in Figure 3b, which used the reporter p3058-T (CEN *TRP1 FUS1pr-lacZ*), a *TRP1*-marked derivative of p3058 [24]. Other *lacZ* reporter plasmids (all CEN *LEU2*, except pPP827, which is 2 μm *LEU2*) were as follows, with the promoters controlling *lacZ* expression indicated in parentheses: p3081 (*FIG1*), p2987 (*KSS1*), p2985 (*PGU1*), and p2988 (*YLR042C*), which were described previously [24]; p3162 (*GPD1*) and p3366 (*YGR043C*), constructed analogous to the preceding reporters to contain 800 and 794 bp of promoter sequence upstream of *lacZ*, respectively; and pPP827 (Ty FRE), which is a *LEU2*-marked derivative of pFRE(Ty1)::lacZ [21].

Microarray experiment gene set definition

MATING gene set: genes induced after both 30 and 60 min of 50 nM α factor were ranked in order of their induction level at 30 min, with the top 20 shown in Figure 3c as follows (from top to bottom): *FIG1*, *YML047C*, *YIL037C*, *YPL192C*, *FUS2*, *YNL279W*, *ASG7*, *AFR1*, *YDR124W*, *FIG2*, *YIL082W*, *CIK1*, *FUS1*, *AGA1*, *YIL080W*, *YCL076W*, *KAR4*, *KAR5*, *HYM1*, and *YOR343C*. HOG gene set: genes induced after both 30 and 180 min galactose induction of the pGAL-SSK2ΔN construct were ranked in order of their induction level at 180 min, with the top 20 shown in Figure 3c as follows (from top to bottom): *YKL161C*, *YNR073C*, *CWP1*, *YLR194C*, *YHR209W*, *YEL070W*, *YIL023C*, *YJL108C*, *PST1*, *SLT2*, *YJL107C*, *GRE2*, *GPD1*, *STL1*, *SRL3*, *YLR042C*, *YGL157W*, *YIL108W*, *DAK1*, and *YPL088W*. Induction by Ssk2ΔN, rather than sorbitol, was chosen to define the HOG set because the induction method, namely, galactose-regulated synthesis of a constitutively active kinase, was most comparable to the Ste11ΔN experiments of primary interest here. Kinetic analysis (Figure S2) suggests that some genes induced by Ssk2ΔN after 180 min may be activated by the PKC pathway as a secondary result of glycerol hyperaccumulation during persistent HOG signaling; therefore, they may serve as indirect, rather than direct, indicators of HOG signaling. Also, Kss1- and Tec1-dependent genes [24, 39] generally were not strongly induced (i.e., 2.5-fold or greater) by any of the galactose-regulated proteins, including Ste11-4 [24], Ste11ΔN, and Ste11ΔN-Ste7; the few exceptions were also induced by either mating pathway activation (e.g., *YIL117C*, *KTR2*, *GFA1*) or HOG pathway activation (e.g., *SRL3*, *DDR48*, *YLR042C*) and thus did not seem pathway specific. Therefore, we could not draw conclusions regarding the effects of the Ste11ΔN fusions on their expression. See the Supplementary material for more information; raw data will be made available on the Rosetta Inpharmatics website (www.rii.com).

Supplementary material

Supplementary material including additional Materials and methods and observations, results with additional Ste11 fusions, and clustering analysis of genome-wide transcription profiling data is available at http://images.cellpress.com/supmat/supmatin.htm.

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References

1. Garrington TP, Johnson GL: **Organization and regulation of mitogen-activated protein kinase signaling pathways.** *Curr Opin Cell Biol* 1999, **11:**211-218.
2. Gustin MC, Albertyn J, Alexander M, Davenport K: **MAP kinase pathways in the yeast *Saccharomyces cerevisiae*.** *Microbiol Mol Biol Rev* 1998, **62:**1264-1300.
3. Madhani HD, Fink GR: **The riddle of MAP kinase signaling specificity.** *Trends Genet* 1998, **14:**151-155.
4. Burack WR, Shaw AS: **Signal transduction: hanging on a scaffold.** *Curr Opin Cell Biol* 2000, **12:**211-216.
5. Elion EA: **Routing MAP kinase cascades.** *Science* 1998, **281:**1625-1626.
6. Choi KY, Satterberg B, Lyons DM, Elion EA: **Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*.** *Cell* 1994, **78:**499-512.
7. Printen JA, Sprague GF Jr: **Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade.** *Genetics* 1994, **138:**609-619.
8. Marcus S, Polverino A, Barr M, Wigler M: **Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module.** *Proc Natl Acad Sci USA* 1994, **91:**7762-7766.
9. Posas F, Saito H: **Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK.** *Science* 1997, **276:**1702-1705.
10. Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ: **MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade.** *Science* 1998, **281:**1668-1671.
11. Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ: **A mammalian scaffold complex that selectively mediates MAP kinase activation.** *Science* 1998, **281:**1671-1674.
12. Feng Y, Song LY, Kincaid E, Mahanty SK, Elion EA: **Functional binding between Gβ and the LIM domain of Ste5 is required to activate the MEKK Ste11.** *Curr Biol* 1998, **8:**267-278.
13. Li W, Han M, Guan KL: **The leucine-rich repeat protein SUR-8 enhances MAP kinase activation and forms a complex with Ras and Raf.** *Genes Dev* 2000, **14:**895-900.
14. Whiteway MS, Wu C, Leeuw T, Clark K, Fourest-Lieuvin A, Thomas

DY, *et al.*: **Association of the yeast pheromone response G protein βγ subunits with the MAP kinase scaffold Ste5p.** *Science* 1995, **269:**1572-1575.
15. Pryciak PM, Huntress FA: **Membrane recruitment of the kinase cascade scaffold protein Ste5 by the Gβγ complex underlies activation of the yeast pheromone response pathway.** *Genes Dev* 1998, **12:**2684-2697.
16. Raitt DC, Posas F, Saito H: **Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway.** *EMBO J* 2000, **19:**4623-4631.
17. Maeda T, Takekawa M, Saito H: **Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor.** *Science* 1995, **269:**554-558.
18. Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, Herz J, *et al.*: **The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2.** *J Biol Chem* 2000, **275:**25625-25632.
19. Whitmarsh AJ, Davis RJ: **Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals.** *Trends Biochem Sci* 1998, **23:**481-485.
20. Yashar B, Irie K, Printen JA, Stevenson BJ, Sprague GF Jr, Matsumoto K, *et al.*: **Yeast MEK-dependent signal transduction: response thresholds and parameters affecting fidelity.** *Mol Cell Biol* 1995, **15:**6545-6553.
21. Madhani HD, Styles CA, Fink GR: **MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation.** *Cell* 1997, **91:**673-684.
22. Sabbagh W Jr, Flatauer LJ, Bardwell J, Bardwell L: **Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation.** *Mol Cell* 2001, **8:**683-691.
23. Roberts RL, Fink GR: **Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth.** *Genes Dev* 1994, **8:**2974-2985.
24. Roberts CJ, Nelson B, Marton MJ, Stoughton R, Meyer MR, Bennett HA, *et al.*: **Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles.** *Science* 2000, **287:**873-880.
25. Ferrell JE Jr, Machleder EM: **The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes.** *Science* 1998, **280:**895-898.
26. O'Rourke SM, Herskowitz I: **The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*.** *Genes Dev* 1998, **12:**2874-2886.
27. Stevenson BJ, Rhodes N, Errede B, Sprague GF Jr: **Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein.** *Genes Dev* 1992, **6:**1293-1304.
28. Cairns BR, Ramer SW, Kornberg RD: **Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase.** *Genes Dev* 1992, **6:**1305-1318.
29. van Drogen F, O'Rourke SM, Stucke VM, Jaquenoud M, Neiman AM, Peter M: **Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo.** *Curr Biol* 2000, **10:**630-639.
30. Bardwell AJ, Flatauer LJ, Matsukuma K, Thorner J, Bardwell L: **A conserved docking site in MEKs mediates high-affinity binding to MAP kinases and cooperates with a scaffold protein to enhance signal transmission.** *J Biol Chem* 2001, **276:**10374-10386.
31. Levchenko A, Bruck J, Sternberg PW: **Scaffold proteins may biphasically affect the levels of mitogen- activated protein kinase signaling and reduce its threshold properties.** *Proc Natl Acad Sci USA* 2000, **97:**5818-5823.
32. Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC: **An osmosensing signal transduction pathway in yeast.** *Science* 1993, **259:**1760-1763.
33. Miller ME, Cross FR: **Cyclin specificity: how many wheels do you need on a unicycle?** *J Cell Sci* 2001, **114:**1811-1820.
34. Bishop AL, Hall A: **Rho GTPases and their effector proteins.** *Biochem J* 2000, **348:**241-255.
35. Robinson MJ, Stippec SA, Goldsmith E, White MA, Cobb MH: **A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation.** *Curr Biol* 1998, **8:**1141-1150.
36. Miyata Y, Adachi S, Mizuno H, Nishida E: **A strategy to make constitutively active MAP kinase by fusing with constitutively active MAP kinase kinase.** *Biochim Biophys Acta* 1999, **1451:**334-342.
37. Whiteway M, Hougan L, Thomas DY: **Overexpression of the STE4 gene leads to mating response in haploid *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1990, **10:**217-222.
38. Sikorski RS, Hieter P: **A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*.** *Genetics* 1989, **122:**19-27.
39. Madhani HD, Galitski T, Lander ES, Fink GR: **Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants.** *Proc Natl Acad Sci USA* 1999, **96:**12530-12535.
40. Reiser V, Salah SM, Ammerer G: **Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42.** *Nat Cell Biol* 2000, **2:**620-627.
41. Farley FW, Satterberg B, Goldsmith EJ, Elion EA: **Relative dependence of different outputs of the *Saccharomyces cerevisiae* pheromone response pathway on the MAP kinase Fus3p.** *Genetics* 1999, **151:**1425-1444.
42. Breitkreutz A, Boucher L, Tyers M: **MAPK specificity in the yeast pheromone response independent of transcriptional activation.** *Curr Biol* 2001, **11:**1266-1271.